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Biophysical Journal 2016, 110(6), 1304–1311.

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DOI link to article:

<http://dx.doi.org/10.1016/j.bpj.2016.01.031>

Date deposited:

01/04/2016

Embargo release date:

29 March 2017



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Investigating DNA radiation damage using X-ray Absorption Spectroscopy (XAS)

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Abstract

The biological influence of radiation on living matter has been studied for years, however several questions about the detailed mechanism of radiation damage formation remain largely unanswered. Among all biomolecules exposed to radiation, DNA plays an important role because any damage to its molecular structure can affect the whole cell and may lead to chromosomal rearrangements resulting in genomic instability or cell death.

To identify and characterize the damage induced in the DNA sugar-phosphate backbone, in this present work we have performed X-ray absorption spectroscopic studies at the P K-edge on DNA irradiated with either UVA light or protons. By combining the experimental results with theoretical calculations, it has been possible to establish the types and relative ratio of lesions produced by both UVA and protons around the phosphorus atoms in DNA.

Introduction

Understanding the interaction between radiation and molecules that make up the building blocks of the human organism is crucial for the design of effective radiation therapy and protection. DNA, being one of the most important components of the human cell, is an influential target of radiation. Therefore most of the fundamental studies focus on the effects caused by radiation on DNA.

Incidences of melanoma skin cancer among the light skinned population is increasing worldwide, and it is well established that the UV radiation range is the most significant part of sunlight that causes it (1). Among UV components of solar light UVA (315–400 nm) comprises about 95%, UVB (280–315 nm) 5%, while UVC (<280 nm) is absorbed by the atmosphere (2). DNA damage arising from UV radiation is resulting in the production of photolesions, including cyclobutane pyrimidine dimers (*CPDs*), 6-4 photoproducts (*6-4PPs*), and their Dewar valence isomers, and by breaking the DNA phosphodiester backbone (3). These photoproducts are characterized by the formation of bonds between two adjacent pyrimidines, which results in a kink in the DNA strand backbone at one of the phosphorus linkages and distortion around it. Photolesions are predominantly produced by direct absorption of UVB photons.

Until recently the UVA range was considered harmless due to the lower energy of the photons, in a spectral region where the absorption of DNA is weak and production of direct photolesions is far less efficient (1). It was believed that the biological effects of UVA are mainly caused indirectly, through the production of reactive oxygen species (ROS) (2). ROS can induce cleavage in DNA strands that are formed typically between one of the oxygen atoms of the phosphate group and 3' or 5' carbon atom of deoxyribose. Radiation-induced DNA termini can include *3'-phosphate*, *3'-phosphoglycolate* and *5'-phosphate* (4) (see Figure 1). The formation of DNA strand breaks by UVA has been reported in a variety of experimental systems, including different forms of DNA as well as in cells (5–8).

Nevertheless, while the absorption of UVA light by DNA is smaller, it is definitely not insignificant (9). The absorption spectrum of the DNA duplex (dA)₂₀·(dT)₂₀ is characterized by a weak, long-wavelength tail which extends over the whole UVA range (10). The formation of *CPDs* by UVA has been detected in numerous studies on UVA-irradiated cells (6, 11, 12), isolated DNA (9, 13) and skin (14) although they are produced 10⁵ times less efficiently than by 254 nm UVC (15). *CPDs* are predominantly formed at thymine-thymine (TT) sites (13). It was believed that the formation of *CPDs* involves mediation of endogenous photosensitizers but further studies showed that *CPDs* are produced also in the absence of other intracellular molecules (13). The predominance for *CPD* formation at TT sites may be explained by the fact that the energy of UVA photons is sufficient to directly excite thymine bases (15). However the detailed mechanism of the damage formation remains unclear.

Understanding the production of *CPDs* and strand breaks by UVA is of significant interest arising from the importance of photoprotection mechanisms. Generally UVA radiation penetrates human skin more effectively than UVB (15) and can reach blood capillaries, affecting peripheral blood cells (7). Based on the assumption that UVA mostly damages DNA through oxidative stress, systemic approaches involving antioxidant supplementation have been proposed. However, the protection from *CPDs* requires complete blocking of the incident photons (13). It is stressed that all photolesions are premutagenic and even their small number can lead to serious mutations and genomic instability in an affected cell (16).

In the case of proton radiation, studies about their interaction with biological systems are primarily driven by potential therapeutic applications in cancer treatment (17). The effectiveness of proton therapy techniques in cancer treatment relates to a superior spatial dose distribution in the patient with minimal lateral scattering in tissue, preventing the unnecessary dose of radiation to be delivered to healthy tissue surrounding the tumor (18, 19). Therefore it has been used to treat a plethora of tumors, including those of the central nervous system, eye, lung, breast and prostate, head and neck as well as sarcomas, and many pediatric cancers (20). Recent experiments on radiation-resistant cancer stem cells, from non-small lung cancer and glioma stem cells, showed that protons exhibit increased cytotoxic effect compared to X-ray photons at the same radiation dose (19, 21). For this reason studies on the effect of proton irradiation on DNA are crucial to enhance the understanding of the mechanisms leading to DNA damage that may be used to increase the effectiveness of radiotherapy. It is also worthwhile to note that protons, as the dominant ionizing particles in interstellar space, are major health risks for astronauts during prolonged space missions (22).

In contrast to UV radiation, protons interact with matter mainly by inelastic Coulombic interactions with electrons. In living cells protons may deposit the energy in DNA or lead to dissociation of water molecules in the surrounding environment. These processes result in direct and indirect DNA damage formation. The dominant damage type, although not necessarily the most dangerous in living organisms, is base damage (23). The second form of

damage consists of single and double strand breaks, often occurring in the formation of clustered damage sites (22, 24). These clustered lesions are difficult to repair and they are thought to be the damage type mainly responsible for both the lethal and mutagenic effects of ionizing radiation (25). As shown by theoretical calculations, the number of strand breaks decreases with increasing linear energy transfer (LET) of protons, which depends on the energy of the particle (26). DNA damage produced by protons is more robust in comparison to X-ray photon-induced damage both qualitatively and quantitatively, with respect to its incidence and complexity. Results of recent experiments suggest that it is mainly a ROS-dependent mechanism (19). Unlike UV radiation, protons do not produce photolesions.

DNA lesions are commonly investigated via gel electrophoresis, comet assays, commercially available specific antibodies and DNA repair enzymes, and more recently high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (HPLC-MS/MS) (27). These methods typically involve DNA degradation, processing, staining or labeling procedures, which by themselves may alter the DNA damage (9). In addition the sensitivity of some of these techniques is low, resulting in inaccuracies in the amount of detected damage. For example T4 endonuclease V, used to specifically recognize *CPDs* (2), has only 60% sensitivity (9). Moreover, gel electrophoresis, typically used in recognition of single- and double-strand breaks e.g. in plasmids irradiated with protons, does not allow detection of DNA fragments that may lead to an underestimation of the measurement, where only a part of the damages is detected (24).

Herein, we use X-ray absorption spectroscopy (XAS) to reveal the types and structure of damaged sites around the phosphorus atoms in the DNA phosphodiester backbone. Importantly, XAS studies are sensitive to both the electronic structure and local geometry around the absorbing atom, in this case the P atom, providing detailed insight into the changes that occur upon DNA damage. Since this is an element specific technique, it does not require pre-concentration of the sample. Until recently, the changes in DNA XAS spectra caused by soft X-ray irradiation have been studied only for the O and N K-edges, and no quantitative information were obtained from these experiments (28, 29). Herein, using XAS measurements at the P K-edge (2.1 keV), we have determined the changes that occur in the phosphodiester DNA backbone after UVA and proton irradiation in order to compare effects of the different radiation types. These changes were compared with theoretical calculations of P K-edge XAS spectra for a variety of damaged DNA structures. The calculations were performed with the FDMNES code (30) that allowed to obtain quantitative results. To the best of our knowledge this work shows the first studies of UVA- and proton-induced DNA damaged structures by means of XAS.

Materials and methods

The experiment was performed on calf thymus DNA (Sigma-Aldrich), which was chosen as a model of the DNA molecule because the numbers of AT and CG pairs are approximately the

same as in human DNA (13). The DNA was irradiated in aqueous solution (10 mg/ml) with UVA radiation or protons. As a reference a non-irradiated DNA sample was used. For the UVA source a UV lamp (Model UVLS-28, UVP, Upland, CA, USA; wavelength 365 nm) was used with delivered power density of about 13 mW/cm² with an irradiation time of 2h that corresponds to a total fluence of 95 J/cm². The sample was irradiated in a glass Petri dish. For particle irradiation we used 70 MeV protons accelerated by the Proteus C-235 cyclotron (The Bronowice Cyclotron Center at the Institute of Nuclear Physics Polish Academy of Sciences in Krakow, Poland). Irradiation was performed in a polypropylene tube. The dose delivered to the sample was 200 Gy.

XAS data were collected at the X07MB (PHOENIX I) beamline of the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland) under vacuum with a double crystal Si(111) monochromator to select the energy of the incoming beam. The sample was placed at 45° to the incident beam. All measurements were performed in fluorescence mode and the X-ray fluorescence was detected using a single-element Si drift diode (Rontec) placed at 90° to the incoming beam to minimize any elastic scatter contribution. The solutions of DNA samples were diluted to 5 mM concentration of phosphorus, which corresponds to 1.55 mg/ml of the sample, and they were flowed during the entire experiment in order to avoid additional radiation damage. The experimental geometry was kept fixed for all samples to allow for identical measurement conditions. The beam size was set to 400 μm x 400 μm and the estimated photon flux on the sample was 10¹¹ photons/s. P K-edge XAS spectra were collected with steps of 5 eV, 0.3 eV, 0.5 eV and 2 eV in the energy ranges 2100-2135 eV, 2136-2160 eV, 2161-2180 eV and 2181-2250 eV, respectively. The photon energy has been calibrated with elemental phosphorus (2145.5 eV). The total acquisition time of a single spectrum was approximately 10 minutes. For each sample 12 spectra were taken and averaged in order to improve the signal to noise ratio. All the spectra were processed using the ATHENA software package (31) and data were normalized to the post-edge region.

Theoretical calculations were performed using the real-space FDMNES code (30), using the finite differences method (FDM) (32) in order to obtain P K-edge absorption spectra. Protein Data Bank (PDB) structures were used to calculate the spectra of undamaged DNA (PDB ID: 1D23) and four kinds of possible damage structures (Figure 1b) that can be produced around the P atoms after irradiation with UVA or protons, namely: 3'-phosphate, 3'-phosphoglycolate, 5'-phosphate and CPD (PDB ID: 1N0K and 1TTD). Structure 1D23 represents a fragment of typical double-stranded B-form DNA, while other structures show the damage sites after strand break and dimer formation.

Results and discussion

The experimental P K-edge XAS spectra are presented in Figure 2. For comparison of the effect of UV and proton irradiation on DNA, the spectra for UVA- and proton-irradiated

DNA samples are compared to undamaged DNA (black traces) in Figure 2a and 2b (upper panels), respectively.

Each experimental spectrum consists of a strong whitenline around 2155 eV and a broad feature in the energy range between 2160 eV to 2180 eV. The whitenline corresponds to the electronic transition of a 1s P electron into an unoccupied orbital formed by the overlap of P sp^3 hybridised with O 2p-orbitals (33). The general shape of the measured XAS spectrum is similar to those measured on PO_4 -type compounds (33). The wide structure centred at 2170 eV is attributed to scattering of the excited photoelectron from the first-shell oxygen atoms in the PO_4 tetrahedra (34). Both structures are thus sensitive to the immediate local P-environment and may be used as a sensitive probe to study local electronic and structural changes. The lower panels of Figures 2a and 2b show the XAS spectral differences (irradiated DNA - undamaged DNA) to highlight the changes. This kind of data analysis is commonly used in the case of pump-probe experiments where spectral differences as small as 1% can be (35). Note that all measurements were performed with the same experimental geometry and concentration to ensure accurate comparison between samples. The error bars represent the standard error of the measured intensity and amounts to about 1%-error of the measured XAS signal. For the difference for UVA-irradiated DNA (Figure 2a bottom) that is about 6 times lower than the measured signal difference at the whitenline position. The difference obtained for the proton damaged sample (Figure 2b bottom) is smaller by a factor of 2 than the one for UVA-irradiated DNA and therefore only 3 times higher than the average error of measured XAS intensity. It has a slightly different shape than the difference spectrum shown in Figure 2a bottom, although it also shows broadly similar characteristic features. In both cases, the experimental results reveal spectral changes in the whitenline intensity and the post-edge multi-scattering feature. These differences can be associated with changes in electronic structure around P atom in the case of damaged structures, such as changes in the P-O hybridization due to bond and angle distortions.

Examples of P K-edge XAS spectra calculated with the use of FDMNES code for the DNA structure of undamaged and the various types of site damage are plotted in Figure 3. For comparison purposes the experimental spectrum of non-irradiated DNA is also shown. In order to show the accuracy of FDMNES calculations in Figure 3 we are also presenting experimental and calculated spectra of adenosine triphosphate (ATP) that is another biologically important compound containing three PO_4 groups. The structure of ATP was taken from PDB.

As shown in Figure 3a and 3b, good agreement between the measured and theoretical spectra for the undamaged DNA and ATP was achieved, both in shape and intensity. Nevertheless, in both cases the part of the spectrum after ~ 2168 eV is not perfectly reproduced by theoretical calculations. This may be due to limitations of the theoretical calculations, such as many body effects (36) or the precision of the Debye-Waller factor (37). Other examples of

FDMNES calculations that reproduced well the experimental data can be found in (38–41). Because the PO₄ geometry in DNA changes slightly depending on the actual P position in the DNA strand, the calculated spectrum represents an average of calculated XAS spectra for all 18 P-sites in PDB structure 1D23. In this DNA structure the typical angle in O-P-O bond is 102.6°. The P-O bond length on a 3' site is 1.59 Å, and for P-O on the 5' site the bond length is 1.61 Å.

In Figure 3c we show the XAS spectra calculated for specific damage sites, with their structure shown in Figure 1 as well as calculated spectrum of free phosphate that also might be present in studied samples. In the case of 3'- and 5'-phosphate the whiteness intensity increases, which is caused by a break in one of the C-O bonds around PO₄. In this case the PO₄ group is now hybridized with only one carbon from the sugar group, leading to larger whiteness intensity. In the case of 3'-phosphoglycolate the whiteness intensity also increases, which is caused by an increase of P-O on the 3' site bond length of 0.03 Å that changes the degree of orbital mixing. For CPD sites we observe a decrease of whiteness intensity and its slight shift towards higher energies that might be caused by the strong changes of P-O geometry. Such a geometry change is caused by dimer formation that leads to an O-P-O bond angle decrease of about 4°. For all damage-type sites, slight changes in the 2155 to 2160 eV range are observed which might be caused by both the change in the hybridization of PO₄ group and by geometrical changes in the O to P bonding, such as different bond lengths and angles, which influence the scattering geometry.

In order to quantify the measured difference signals, the theoretical spectra depicted in Figure 3c were then used in a least-square fitting procedure to determine the type of damage sites and their relative contributions. The result of the fit for UVA-irradiated DNA sample (R² value of 0.95) is presented in Figure 4 by the black solid line. The contribution of 3'-phosphate, CPD and 5'-phosphate damage sites to the measured XAS difference are shown as blue, green and red solid lines respectively. The 3'-phosphoglycolate site and free phosphate were also used in the fitting procedure, however the determined contributions were found to be well below the statistical noise (<0.1%) and therefore are not plotted in Figure 4. As shown in Figure 4, the signal difference at the position of the whiteness could be reproduced within the experimental error, including the small feature at 2153 eV. In the energy range 2155 eV to 2162 eV the overall shape is well reproduced, but the intensity of the difference is underestimated by about 30 %.

Next, we applied the same fitting protocol to quantify the changes observed in proton-irradiated DNA. The results are presented in Figure 5. All five calculated reference spectral differences were used in the fitting process, nevertheless the contribution of 3'-phosphoglycolate, CPD and free phosphate were below 0.1% and they are not shown in Figure 5. The negligible contribution from CPDs is reasonable because they are produced specifically by UV (42) and thus the only forms of damage detected by us in case of protons

are strand breaks. As mentioned previously, strand breaks may be formed by proton irradiation both directly and indirectly. The colour representation of the remaining components is the same as in Figure 4. The fit quality is not as good as that for the UVA irradiated sample ($R^2=0.8$) however the general shape of the experimental difference was reconstructed by the chosen theoretical spectra within the obtained errors.

The summary of both fit results, together with error values, are presented in Table 1.

Table 1. Fit results for experimental spectral differences obtained for UVA- and proton-irradiated DNA spectra, fitted with the spectra of different lesion structures calculated using the FDMNES (30) code together with error values and R^2 for each fit.

| Lesion Type | Relative ratio | Error |
|---------------------------------------|----------------|--------|
| UVA ($R^2=0.95$) | | |
| <i>3'-phosphate</i> | 56 % | 6.2 % |
| <i>CPD</i> | 32 % | 12 % |
| <i>5'-phosphate</i> | 12 % | 5.8 % |
| <i>3'-phosphoglycolate</i> | < 0.1 % | |
| <i>free phosphate</i> | < 0.1 % | |
| Protons ($R^2=0.8$) | | |
| <i>3'-phosphate</i> | 74 % | 17.6 % |
| <i>5'-phosphate</i> | 26 % | 19.6 % |
| <i>3'-phosphoglycolate</i> | < 0.1 % | |
| <i>CPD</i> | < 0.1 % | |
| <i>free phosphate</i> | < 0.1 % | |

As can be noticed the uncertainty of the fit results is quite high due to the large value of errors and fit results should be used mostly to identify the degradation products and their approximate ratio. Based on the fitting procedure, the results show that for the UVA-irradiated samples most of the damage sites are strand breaks, of which the *3'-phosphate* termini is most frequent (56%), compared to *5'-phosphate* (12%). In case of UVA, the mechanism of strand break formation is indirect. Indirect strand breaks are produced mainly by hydroxyl radicals (2). *CPDs* represent the smaller part of UVA-generated lesions (32%) which is understandable due to very low absorption of UVA by DNA (43). Nevertheless, it has been proposed that DNA in aqueous solution has improved mobility and a larger backbone flexibility that increases the number of reactive conformations that can produce *CPDs* (44). Although some of the previous experiments indicated that *CPDs* are the dominant form of lesions in UVA-irradiated DNA, these studies shows a large discrepancy especially

in the ratio of *CPDs* to strand breaks (2, 9, 27). In contrast, the experiment performed on *Bacillus subtilis* spore DNA showed that sunlight with wavelengths >325 nm induced both double-strand breaks and single-strand breaks while *CPDs* in spore chromosomal DNA were not detectable (8). As it might be concluded from these experiments, the resulting number of damage types depends on the irradiation conditions and irradiated sample.

The fitting of the difference obtained for proton-irradiated DNA shows that *3'-phosphate* is also the dominant type of damage formed during proton irradiation. It represents 74% of all strand breaks produced by protons, while the remaining 26% was *5'-phosphate*. It indicates that the bond between the 5' carbon atom of deoxyribose and oxygen in the phosphate group, is the most likely to break after both UVA and proton irradiation. This is corroborated by the theoretical structure of DNA where this bond is about 0.02 Å longer than the bond between 3'C and O. Most of the methods used to study proton damaged DNA show a large number of both signal and double strand breaks (22, 24). Nevertheless, they do not give any information on which bond is predominantly broken to produce these breaks. That information can be obtained only by the methods sensitive to the structure of molecules, such as XAS, which also provides detailed information on the changes in electronic structure around the damaged sites along the sugar-phosphate backbone.

Conclusions

The key to more effective radioprotection and radiotherapy is to understand the exact mechanism of radiation interaction with biomolecules, in particular to obtain the precise structure of the different forms of damage and their relative ratios. We have demonstrated a novel approach, based upon X-ray spectroscopy, to investigate the structure of DNA lesions caused by irradiation by either UVA light or protons. Our approach provides information not only about the damage types but also about changes in electronic structure around the phosphorus atoms associated with each damage type, which can help to establish the possible mechanisms involved. Moreover, the applied method provides us with approximate quantitative information as to which bond in the sugar-phosphate backbone most likely will be broken after irradiation. Nevertheless the quantitative results are not as precise as would be desired for a quantitative method and for precise damage population estimates XAS measurements can be supplemented with additional measurements using other techniques. The experimental procedure used does not require any sample chemical preparation or treatment, avoiding any additional sample preparation complications affecting the conclusions. The measured X-ray absorption spectra are sensitive to the changes in the local neighbouring structure, as in the case of strand breaks, but also to changes in geometry around the P atom, as in case of *CPDs*. By combining the experimental results with full-potential X-ray scattering calculations using the FDMNES code it was possible to determine that strand breaks are the main lesions caused in DNA by both UVA and protons, with *3'phosphate* being the dominant form of termini, indicating the 5'C-O bond cleavage as the

most likely location for strand breaks. The *CPDs* were found to be a minor product of UVA interaction with DNA, nevertheless their presence confirm the direct absorption of UV light with wavelengths of about 365 nm. This approach can easily be extended to identifying other forms of DNA damage caused by, for example, UV-B and UV-C or chemical agents used for chemotherapeutic purposes.

Author contributions

J.C.-M., J.Sz., C.J.M., E.L. and J.S. designed the research. J.C.-M., J.Sz., C.J.M., E.L. and W.M.K. performed the experiment. T.H. and C.B. worked on the technical development of the experimental setup and supported the experiment. J.C.-M., J.Sz. and T.J.P. performed theoretical calculations. R.A. and W.M.K oversaw the study and contributed critical discussion. J.C.-M. wrote the manuscript with contributions from J.Sz., C.J.M., J.S. and T.J.P. All authors read and accepted this manuscript.

Acknowledgments

We acknowledge Swiss Light Source at Paul Scherrer Institute for granting the beamtime in the proposal 20140269. The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement n.º 290605 (COFUND: PSI-FELLOW).

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Figure captions:

Figure 1. a) Chemical structure of the fragment of DNA strand. Arrows indicate the bonds that can be broken and lead to 1: 3' – termini; 2: 5' – termini. b) Structures of the four lesion types: 3'-phosphate, 3'-phosphoglycolate, 5'-phosphate and CPD. Distorted PO₄ groups are highlighted with orange circles.

Figure 2. a) top) Phosphorus K-edge x-ray absorption spectra of intact and UVA-irradiated aqueous DNA samples; bottom) P K-edge XAS difference signal between the spectrum of damaged and reference DNA sample. b) top) Phosphorus K-edge x-ray absorption spectra of undamaged and proton-irradiated DNA samples; bottom) P K-edge XAS difference between the spectrum of damaged and undamaged DNA sample.

Figure 3. a) Comparison of P K-edge XAS spectrum of undamaged DNA calculated in FDMNES together with the experimental spectrum of non-irradiated DNA. b) Comparison of P K-edge XAS spectrum of ATP calculated in FDMNES together with the experimental spectrum. c) P K-edge XAS spectra for different damage sites calculated by FDMNES.

Figure 4. The experimental difference of P K-edge XAS obtained for UVA-irradiated DNA sample fitted with the theoretical spectra.

Figure 5. The experimental difference of P K-edge XAS obtained for proton-irradiated DNA sample fitted with the theoretical spectra.